FLUORESCENCE HYBRIDIZATION ASSAY BASED ON CHITOSAN-LINKED SOFTARRAYS

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ABSTRACT

The work to be presented is examining the development of a hybridization-based biosensor. The design of a high throughput assay based on standard laboratory instrumentation and 96-well microtiter plates will be discussed as well as a highly sensitive sensor using a novel tapered fiber optic probe. The immobilization chemistry learned in the 96-well plate will be implemented on the fiber optic sensor. The high throughput assay is based on the immobilization of single-stranded, synthetic oligonucletides inside a 96-well plate utilizing chitosan and glutaraldehyde. Chitosan is a biopolymer with abundant primary amines for attachment chemistry and is easily deposited as thin layers on surfaces. Hybridization can take place inside the wells with a complementary analyte sequence followed by a fluorescent, labeled signaling probe to form a sandwich assay. The hybridized wells can be interrogated with any bench-top fluorometer with a microwell plate reader.

INTRODUCTION

There is a great need for high throughput and sensitive sensors for genetic analysis. These sensors can be used for varied purposes from monitoring gene expression in organisms to speciation of possible pathogens. An instrument capable of these tasks would be a great benefit for food and water safety, medical diagnostics and defense of military and civilian populations from biological threats.

There are many recognition elements, which have been utilized in biosensor design. Nucleic acid hybridization assays have become popular techniques. Hybridization assays have been developed to interrogate samples for multiple analytes from a single sample.[1,2] There is also a great interest in employing fiber optic based systems for varied tasks from the analysis of gene expression to detection of biological pathogens.[3-5] The goal of the research is to design a sensitive biosensor by the use of evanescent field fluorescence spectroscopy, which has been described for biosensors as well as other organic matrices.[6,7]

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Tapering the distal end of a fiber optic probe enhances the evanescent field around the fiber. This evanescent field can be used to both excite and collect the fluorescence from dye molecules close (~100-nm) to the fiber. This will reduce the background by eliminating fluorescence from unbound dye molecules in the sample matrix. The enhancement of the evanescent field and limiting the sample area to very close to the fiber surface can be used to increase fluorescent signal from a hybridized sandwich assay attached to the surface without increasing the background due to unbound, dye-labeled oligonucleotide probes in solution.

In order to perform hybridization techniques in this format the nucleic acid sequences need to be immobilized on a substrate. The optical properties of this substrate should also not be disrupted. A common approach to immobilize biomolecules on surfaces involves the modification of the surface with amine functionality.[8] Typically, this is achieved with aminosilanization or coating with a polyamine such as polylysine. Testing of linkage chemistry, buffer selection, and stability of hybrids requires a high throughput method. The typical high throughput hybridization technique is based on arrays spotted on microscope slides that require very expensive hardware for deposition and reading of the arrays. It was determined that a more cost effect technique needed to be developed to screen the chemistries. A system was designed where chitosan, a biopolymer with abundant primary amines for attachment chemistry, modifies the interior of a 96-well microtiter plate. These plates can be interrogated in a standard benchtop spectrofluorimeter for a variety of dye labels.

Chitosan was selected for the surface modification for several reasons. First, it is easily deposited onto surfaces due to its solubility at low pH and insolubility when the pH is raised close to neutral.[9] Chitosan is also transparent to UV and visible light therefore it will not be detrimental to the optical detection scheme. The amine groups on chitosan are also highly reactive relative to other polyamines (e.g. polylysine) and useful for attachment of biomolecules such as proteins.[10-12] Finally, chitosan is inexpensive, readily available and safe to handle.

We have examined the colvalent coupling of single-stranded, synthetic DNA oligonucletide (ssDNA) sequences to the chitosan modified surfaces. This serves as a recognition site to hybridize a complementary, dye-labeled oligonucletide showing that the formed “softarrays” are suitable for DNA hybridization assays. This was achieved by utilizing glutaraldehyde, a routinely used homobifunctional cross-linker, to attach amine-terminated oligonucletides to the surface amine groups.[6, 13]

The first step, to insure the chemistry is functioning as designed, was to immobilize 4’-((aminoacetamido)methyl)fluorescein (AF) on the chitosan modified 96-well plate. Upon successful linkage of AF, single-stranded, fluorescein-labeled, amine-terminated ssDNA was immobilized and inspected. Finally, the amine-terminated ssDNA was hybridized with a fluorescein-labeled complement providing the proof of concept for this project. Figure 1 illustrates this stepwise proof of concept.

We will discuss chitosan coating conditions, cross-linking with glutaraldehyde, immobilization of the ssDNA, hybridization and nonspecific binding issues on the “softarrays”.

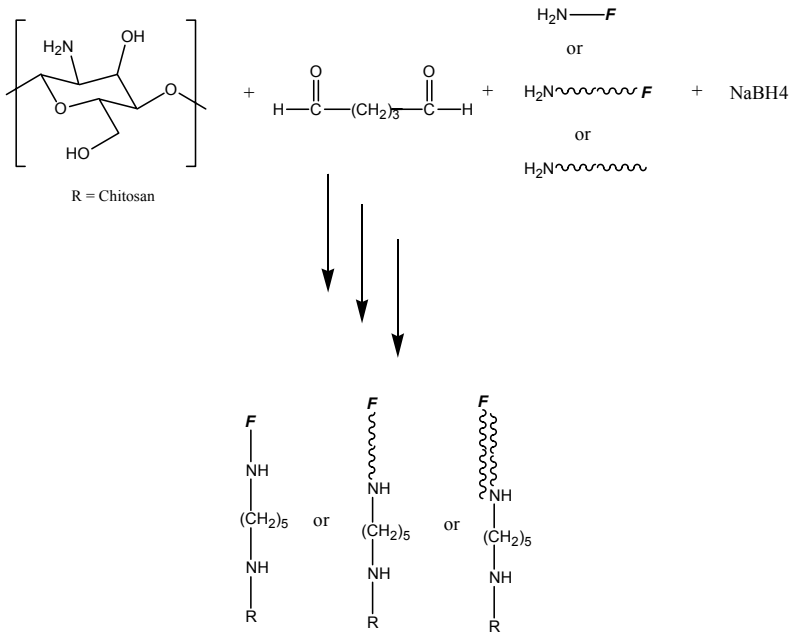

Figure 1. Diagram of covalent coupling of AF, modified ssDNA and hybridization.

EXPERIMENTAL METHODS

Chitosan-coated, 96-well plates were prepared by first dissolving appropriate quantities of chitosan flakes in dilute (0.5% v/v) HCl. Films of chitosan were prepared by adding 50-300 µl of 0.1 – 1.5 (w/v) % chitosan solution to each well of 96-well plates (cell-culture treated, transparent). The plates were then dried in an oven overnight at 45°C and then the films were neutralized by immersion in 1 M NaOH. After neutralization, the films were thoroughly washed with autoclaved ddH_2O and saturated sodium citrate with magnesium chloride (SSC+Mg) buffer. The plates were stored at 4°C with buffer in the wells until use.

Each well coated with chitosan was rinsed with buffer at least twice. An aliquot of 1%(v/v) glutaraldehyde solution in buffer was added to each well and incubated for 1 hr. After extensive washing with buffer, AF or fluorescein-labeled ssDNA with or without 5' end amine group at 25 µg/ml were added and incubated overnight at 4°C.

DNA oligonucleotide sequences of *E. coli* genes, *dnaK* and *groEL*, are shown in Table 1 with their complementary target sequences with fluorescein labels. These 20-mer sequences were located near the 3' ends of the *dnaK* and *groEL* genes and were selected because the specific regions had little homology to other *E. coli* genomic sequences.

Table 1. List of oligonucleotide sequences and modifications

ssDNA oligonucleotide	5' end modification	Sequence	3' end modification
amine terminated *dnaK*	NH_2	CTT TCG CGT TGT TTG CAG AA	(none)
amine terminated *groEL*	NH_2	TTT TTC GGC AGG TCG GTA AC	(none)
Fluorescein labeled target *dnaK*	FITC	TTC TGC AAA CAA CGC GAA AG	(none)
Fluorescein labeled target *groEL*	FITC	GTT ACC GAC CTG CCG AAA AA	(none)
Fluorescein labeled and amine terminated *dnaK*	NH_2	CTT TCG CGT TGT TTG CAG AA	FITC

Underlined sequences represent 5 complementary portion between *dnaK* and *groEL*.
* FITC : fluorescein isothiocyanate

After overnight incubation, each well was extensively washed with buffer and treated with Tris-HCl buffer to consume any unreacted aldehyde groups. After thorough rinsing, a solution of sodium borohydride powder dissolved in buffer was added to each well. The sodium borohydride solution was incubated in the wells to reduce the Schiff base resulting from the reaction of aldehyde and amine groups. After this reaction, the yellowish color representative of a Schiff base disappeared and the background fluorescence signal dropped to the initial ~8 to 12 fluorescence intensity (FI) units. Each well was then further rinsed with a urea solution (pH ~7.5) and rinsed again with buffer extensively to remove nonspecifically bound ssDNA. At this stage, the chitosan gels coated with 5' end amine terminated oligonucleotides were ready for hybridization experiments. All room temperature reactions and rinses were performed with gyratory shaking. Overnight incubation at 4°C for ssDNA or AF immobilization was performed in static solutions.

Hybridization was performed by adding an aliquot of 5' end fluorescein-labeled ssDNA in SSC+Mg buffer in each well and by incubating for 1 hour at room temperature. Fluorescence was measured at each step before and after extensive washes with SSC+Mg buffer, a urea solution, and a subsequent SSC+Mg buffer wash.

A Perkin-Elmer LS55 fluorescence spectrometer was used for all fluorescence measurements. The excitation wavelength was set at 494nm and emission wavelength at 520nm. Initial background signal (after glutaraldehyde treatment but prior to coupling) was subtracted from total FI for each well. More specifics on the materials and experimental procedures are to be published.[14]

RESULTS AND DISSCUSSION

In preliminary studies we observed that AF, our reporter for coupling, does not bind to microtiter plates that had not been coated with chitosan. Additionally, under all wash/immobilization conditions we observed that dried chitosan films bound more tightly to cell culture treated microtitier plates than to untreated plates. Thus all studies reported here were performed with cell-culture treated microtiter plates.

To examine whether the deposition, activation, and coupling steps were robust, chitosan films were created in multiple ways. Specifically, both the volume and concentration of chitosan solutions added to the wells were varied. After drying, neutralizing, and activating with glutaraldehyde, the activated chitosan films were exposed to AF. In Figure 2, the FI was plotted against the total chitosan. The trend shows a saturation behavior where the FI increased with the total chitosan. All the data collapsed to a single curve, demonstrating the robustness of the procedure with which the "softarrays" are created and labeled.

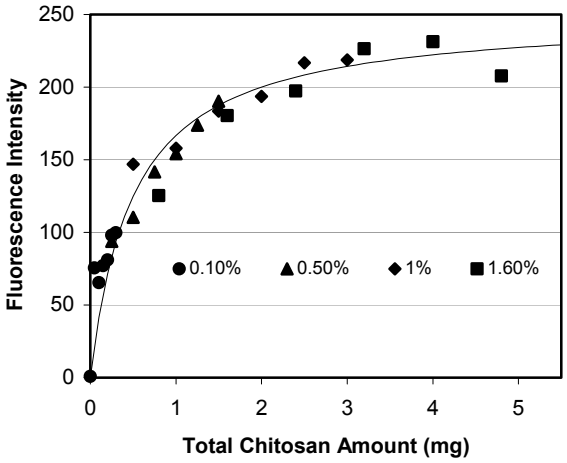

Figure 2. Fluorescence versus total total chitosan concentration deposited in 96-well plates.

At the higher chitosan levels, in addition to the observed saturation in fluorescence, we observed that the films became brittle upon reaction with glutaraldehyde and variance in fluorescence from well to well increased. Therefore plates were prepared with 70 μl of 1.5 %(w/v) chitosan solution for each well for further studies.

To examine the covalent coupling of ssDNA to the chitosan films, ssDNA with amine or fluorescein terminal modification were reacted with the chitosan film. Figure 3 shows the resulting fluorescence after immobilization of AF, H_2N-5'-ssDNA-3'-FITC, FITC-5'-ssDNA and H_2N-5'-ssDNA onto chitosan films. Treatments were run with and without glutaraldehyde in order to differentiate covalent coupling from nonspecific adsorption. The fluorescence resulting after both the SSC+Mg buffer wash and the subsequent urea wash are shown.

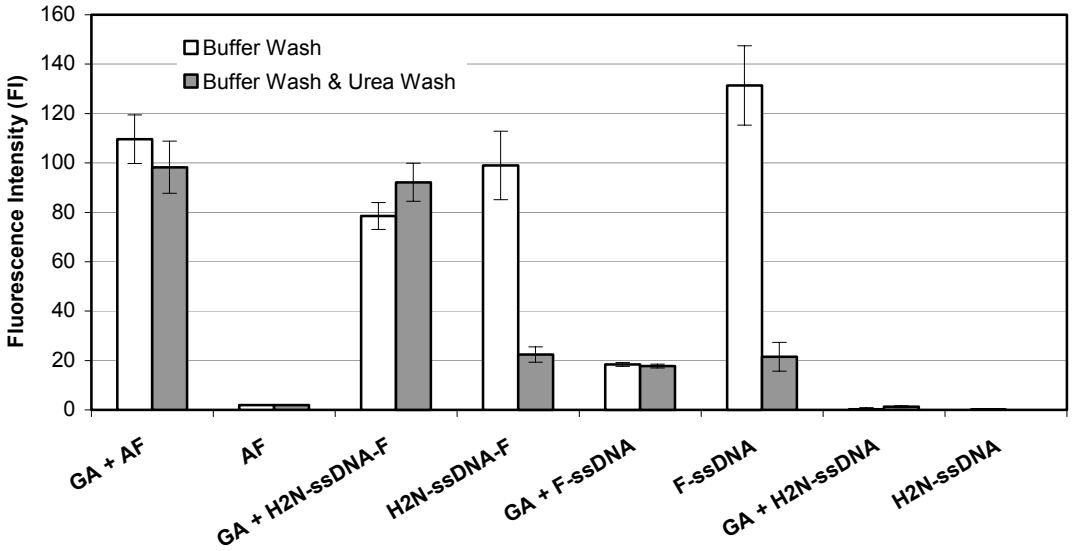

Figure 3. Immobilization of single stranded DNA onto chitosan film for various chemistries prior to and after a urea wash.

The first two sets of bars demonstrate that the low-molecular weight reporter AF binds readily (FI > 100) with glutaraldehyde and that the bound AF is not washed away by either the SSC+Mg buffer or urea wash steps. Additionally, AF will bind to chitosan via non-specific ionic or hydrogen bonding interactions, but this is readily removed by SSC+Mg buffer wash. Importantly, when 0.1M $MgCl_2$ was included in the buffer (SSC+Mg buffer), there was lower nonspecific binding (131 FI, compared to 300 FI – data not shown) between chitosan and FITC-labeled ssDNA.

Next, 5' amine-terminated and 3' FITC-labeled oligonucleotide (H2N-ssDNA-F) was used to analogously demonstrate the covalent immobilization of ssDNA onto glutaraldehyde-activated chitosan films. When glutaraldehyde was added to activate the chitosan film, 80 – 90 FI was consistently obtained and no significant difference in FI was observed between the SSC+Mg buffer and/or urea wash steps. When glutaraldehyde was not added, the fluorescence was appreciable (~100 FI) when the films were only washed with the SSC+Mg buffer. Thus we observed significant nonspecific binding between the un-activated chitosan films and ssDNA. After a urea rinse the FI dropped to ~22 FI indicating that the urea rinse disrupted this apparent affinity, removing non-specifically bound ssDNA.

Fluorescein labeled oligonucleotides that were not terminated with amine groups (F-ssDNA) were also studied. These F-ssDNAs served as controls as they should not covalently couple to the glutaraldehyde-activated chitosan films. When F-ssDNA was contacted with glutaraldehyde-activated chitosan films and then washed, low but non-zero FI was observed and the urea wash had no effect on the interaction. When F-ssDNA was contacted with chitosan films that had not been activated with glutaraldehyde, significant fluorescence was observed, but this was readily removed with the urea wash.

Finally, a 5' end amine terminated ssDNA was coupled to chitosan films, and only minimal fluorescence was detected irrespective of the presence of glutaraldehyde, as expected. In addition, this was completely removed upon washing in SSC+Mg buffer and urea. In summary, these results clearly demonstrate that ① nonspecific binding of DNA to chitosan is appreciable, ② glutaraldehyde reduced such nonspecific binding dramatically while serving as a bridging molecule for covalent coupling to

amine terminated molecules and ③ the background fluorescence associated with covalently anchored ssDNA probes, used for subsequent studies of DNA hybridization, is minimal.

The experiments above used NH_2-ssDNA-FITC to validate anchoring of ssDNA onto chitosan films. As indicated earlier, our ultimate goal was to immobilize amine terminated ssDNA without 3' end FITC label, to investigate the hybridization with 5' end fluorescein labeled complementary target ssDNA.

Hybridization reactions were performed wherein 5' end FITC labeled ssDNA complementary to the bound probe ssDNA was incubated for 1hr at room temperature. After hybridization, extensive washing with SSC+Mg buffer was followed by a urea rinse to remove nonspecific binding, as noted previously. The resulting fluorescence measurements from a 20 base *dnaK* target sequence complementary to the bound unlabeled ssDNA probe are shown in Figure 4. Significantly, the SSC+Mg buffer and urea solution did not wash away the fluorescence resulting from the hybridization. More importantly, the fluorescence was linear within the range of target sequence concentrations tested (0.73-6.6 μM). Although the final results are shown in the figure, the fluorescence was nearly constant before and after urea wash, suggesting that the nonspecific binding sites of chitosan were already occupied or otherwise altered by glutaraldehyde and the amine-terminated ssDNA during probe surface preparation, thus producing a much lower background signal than was found in the previous section.

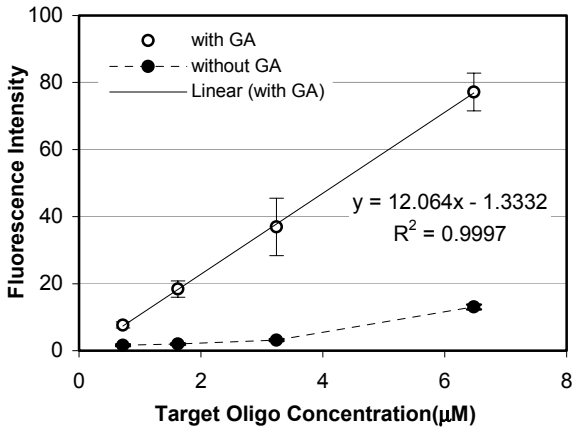

Figure 4. Hybridization of fluorescein labeled *dnaK* ssDNA with complementary ssDNA immobilized onto chitosan films.

As shown by the solid circles, chitosan films that were not activated with glutaraldehyde were also incubated with amine terminated ssDNA and tested as a negative control for hybridization. The resulting fluorescence demonstrated that there was minimal nonspecifically bound probe available for hybridization. Further, our results indicated that sub-nanomolar quantities of target molecule were easily detected with the current format. That is, 140 picomoles of *dnaK*-specific ssDNA were detected from 200μl samples in 0.73μM concentration.

To test whether the hybridization was sequence-specific, and as a second negative control, mismatched target sequences were FITC-labeled and incubated with the ssDNA probes. Chitosan surfaces coated with a 5' end amine terminated ssDNA of 20 bases from *groEL* gene sequence in *E. coli* was prepared and tested identically to the *dnaK* target above. The mismatched pair, *groEL-dnaK* (probe-target), yielded much lower fluorescence than the matching pair *dnaK-dnaK* with the same target oligo concentration. This result, with more than 5-fold increase in fluorescence over the mismatched control (18.4 FI, match versus 3.6 FI, mismatch), indicates that the observed fluorescence was sequence specific.

CONCLUSIONS

We believe that the pH dependent solubility and reactivity (i.e. low pKa) of chitosan presents an attractive scaffold upon which to build hybridization detection chemistries that, at present, are formulated in "softarrays" in the bottom of 96-well plates, but could be built on other surfaces based on the controlled deposition of chitosan.

It was demonstrated, by collapsing the data onto a single curve, some chitosan amine groups remained unreacted with excess AF. The work also indicates that there is considerable flexibility in the deposition protocol that might be exploited to tailor the sensitivity of the detection assay. More importantly, the observation that results from these studies suggests that the steps required for deposition, activation, and coupling are robust.

There was significant nonspecific binding between ssDNA and chitosan films. Because we first demonstrated coupling of AF to chitosan, the AF residue itself was not the cause of the nonspecific binding of labeled ssDNA. It is also interesting to note that before rinsing with urea, glutaraldehyde-activated chitosan films had much less nonspecific binding with non-amine terminated ssDNA than untreated chitosan films (20 FI vs. 131 FI). This clear difference in fluorescence after the buffer wash due to the presence and/or absence of glutaraldehyde may suggest that the amine group from chitosan is responsible for these nonspecific interactions. Thus, in order to utilize ssDNA for hybridization, amine groups from chitosan should be blocked to maximize hybridization capacity of the probe ssDNA, since bases that hydrogen bonded with chitosan may not participate in hybridization efficiently. Our scheme has enabled this by first modifying amine groups from chitosan through the glutaraldehyde crosslinking. Hybridization experiments where glutaraldehyde was used showed, a reduction the nonspecific binding of FITC-labeled, target ssDNA.

This report describes first use of chitosan as a biopolymeric scaffold to assay nucleic acid hybridization through covalent coupling of ssDNA. A combinatorial approach using a 96-well microtiter plate format enabled rapid and parallel evaluation of solution chemistries and conditions for achieving satisfactory performance. By taking advantage of abundant and reactive amine groups that already exist in chitosan; this technique is simple to carry out with common equipment and without the need to deal with hazardous materials such as strong acids and/or silanizing reagents that are required for surface treatments otherwise. This technique is also likely scaleable to enable high throughput screening of DNA/RNA hybridization.

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